Alignment of Rat Cardionatrin Sequences with the Preprocardionatrin Sequence from Complementary DNA

Abstract. *Mammalian atria contain peptides that promote the excretion of salt and water from the kidney. When rat atrial tissue is extracted under conditions known to inhibit proteolysis, four natriuretic peptides, cardionatrins I to IV, are consistently isolated. These peptides derive from a common precursor, preprocardionatrin, of 152 amino acids, whose sequence was determined by DNA sequencing of a complementary DNA clone. Amino acid sequencing located the start points of cardionatrins I, III, and IV in the overall sequence. Cardionatrin IV most closely resembles procardionatrin because it begins immediately after the signal sequence at residue 25. Cardionatrin III begins at residue 73, and cardionatrin I, sequenced previously, begins at residue 123. Compositional analysis indicated that each of these cardionatrins extends up to tyrosine at position 150 but lacks the terminal two arginine residues.*

Several laboratories have recently reported the isolation and amino acid sequences of peptides from mammalian atria that are potent diuretic, natriuretic, and vasodilatory agents (*1–4*). This recent work substantiated previous studies (*5, 6*) that showed that atrial extracts contain a number of these peptides which, although differing in molecular weight, have the same biologic activity. The peptides were referred to collectively as atrial natriuretic factor (*7*). All the peptides sequenced so far are either truncated or extended versions of a 28-residue disulfide-looped structure (designated cardionatrin I) whose sequence was reported initially from our laboratory (*1*).

Cardionatrin I is one of the most abundant of the native natriuretic peptides in atrial muscle when tissue extractions are carried out under conditions known to inhibit proteolysis (*8*). Under such conditions, we have not found the multitude of different peptides reported (*2, 3*) that differ in amino acid sequence from cardionatrin I by a few amino acids. On the contrary, we have consistently observed only three other biologically active peptides—cardionatrins II, III, and IV—all of which have larger molecular weights than cardionatrin I. All the biologically active atrial peptides have been shown to derive from a common precursor (*9–12*), but it remains to be determined which of the peptides isolated are the precursor's natural cleavage products.

We now report that cardionatrin IV, the largest peptide, is the proprotein precursor of cardionatrin I. This discovery has allowed us to establish the junction between the signal peptide and the proprotein, a feature that had been postulated from the complementary DNA (cDNA) sequence. The amino terminal sequence of the proprotein has a high degree of homology with cardiodilatin, a vasodilating peptide isolated from pig heart (*13*); this finding raises the possibility that different functions are associated with different parts of the protein. In addition, we have independently established the sequence of a cloned cDNA for the rat cardionatrin precursor.

Rat atria were extracted with acid as described (see legend to Fig. 1). After fractionation of the atrial extract on Bio-Gel P-10 (Fig. 1a), diuretic activity was found in four broad regions (I to IV) of differing molecular weights. Pooled fractions from each of the areas I, II, III, and IV were processed separately by reversed-phase high-performance liquid chromatography (HPLC) (Fig. 1, b to e). Purified, homogeneous cardionatrins I, III, and IV were obtained by these and additional processes as described (see legend to Fig. 1).

Cardionatrin I was identical to the peptide isolated and sequenced previously (*1*). Cardionatrin IV had a molecular weight of approximately 19,000 as revealed by urea–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). The primary structure of the first 30 residues of this peptide was determined by stepwise Edman degradation of the unmodified protein by means of a gas-phase sequencer (Applied Biosystems model 470A) (*14*).

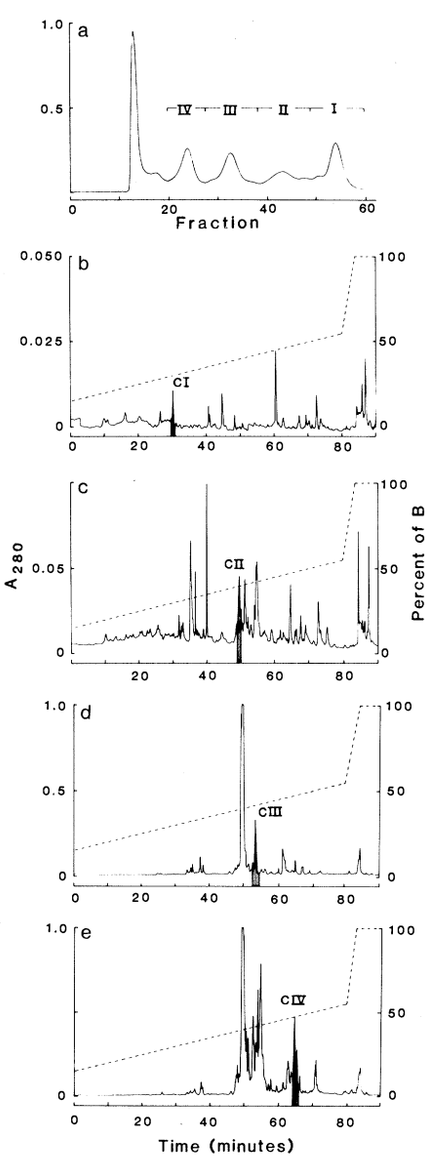

Fig. 1. Gel-permeation chromatography of atrial extracts (a) and the first purification step by HPLC of cardionatrins I (b), II (c), III (d), and IV (e). One thousand frozen rat atria were ground together with solid CO_2 chips and homogenized (Polytron) in 10 volumes (by weight) in 1.0*M* acetic acid, 1.0*N* HCl, and 1 percent NaCl. After centrifugation of the extracts, the supernatants (20-ml portions) were passed through wetted Sep-Pak (Waters) cartridges. Each cartridge was washed with 20 ml of 0.1 percent trifluoroacetic acid (TFA) and eluted with 3 ml of 80 percent acetonitrile in 0.1 percent TFA. The eluted material was combined, freeze-dried, dissolved in 5 ml of 1.0*M* acetic acid and 1 percent NaCl, and fractionated on a BioGel P-10 column (2.5 by 40 cm) equilibrated with the same solution. Fraction pools I, II, III, and IV (a) were processed further by HPLC by pumping them directly into two serially connected Vydac C_{18} columns (10 by 250 mm) that had been equilibrated with 12 percent acetonitrile in 0.1 percent TFA. Elution was carried out at 3.0 ml per minute with acetonitrile gradients (12 to 44 percent) containing 0.1 percent TFA. These gradients were achieved by varying the input of the organic pump (percent of B, b to e), which delivered an 80 percent (weight by volume) acetonitrile solution in 0.1 percent TFA. Elution times for cardionatrins I, II, III, and IV were determined by the rat bioassay (*7*) and are indicated as CI (b), CII (c), CIII (d), and CIV (e), respectively. Cardionatrins I and II were isolated as described in (*1*) and by further purification in similar acetonitrile gradients containing 0.13 percent heptafluorobutyric acid as counter-ion. Cardionatrins III and IV were purified further by high-performance cation-exchange chromatography (Spherogel TSK, 4 by 300 mm; Altex) with a gradient (over 60 minutes at 1.0 ml per minute) of 0.010*M* to 1.0*M* ammonium formate buffer (*p*H 6.5) containing 10 percent acetonitrile. All cardionatrins went through a final purification step performed in a Vydac C_{18} column (4.6 by 250 mm), eluted at 1.5 ml per minute with acetonitrile gradients containing 0.1 percent TFA.

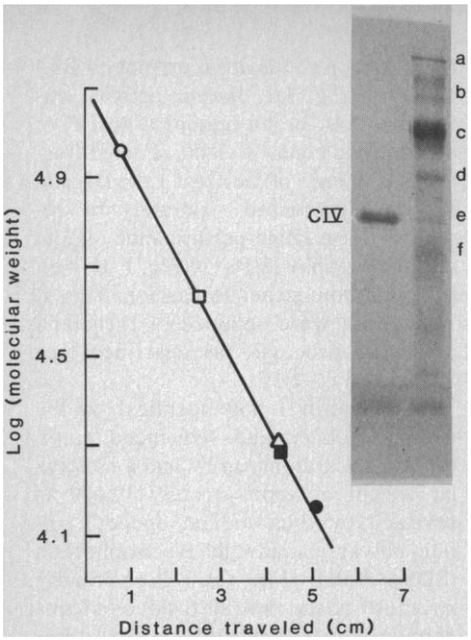

Fig. 2. Urea–SDS-PAGE of cardionatrin IV. The system used was essentially that of Laemmli (*19*). The molecular weights of standard proteins are (a) 92,500 (phosphorylase B); (b) 66,200 (bovine serum albumin); (c) 45,000 (ovalbumin); (d) 31,000 (carbonic anhydrase); (e) 21,500 (soybean trypsin inhibitor); and (f) 14,300 (lysozyme). The standard curve was constructed with ovalbumin (○), carbonic anhydrase (□), soybean trypsin inhibitor (△), and lysozyme (●). Cardionatrin IV is indicated by (■).

The sequence was identical to residues 25 to 54 of the anticipated preproprotein sequence from a cloned cDNA sequence determined by us (Fig. 3) and by others (*9–12*). Both the molecular weight and amino terminal sequence of cardionatrin IV are consistent with its being procardionatrin, but the amino acid composition (Table 1) suggests that it lacks the two carboxyl terminal arginine residues.

The amino terminal sequence of cardionatrin IV beginning with asparagine is the predicted cleavage point for the removal of the signal sequence according to the observations of Perlman and Halverson (*15*). Before these observations were made, the junction of the signal peptide and proprotein had been predicted correctly by Yamanaka *et al.* (*9*) and Maki *et al.* (*10*). The analogous position in the human natriuretic factor precursor was shown to be a cleavage point by the isolation and characterization of γ-human atrial natriuretic polypeptide (*11*), which is the human analog of cardionatrin IV. In addition, the amino terminus of cardionatrin IV is in register with, and matches almost exactly, the amino terminal sequence of cardiodilatin, a peptide with smooth muscle relaxant activity isolated from porcine atria (*13*). In cardiodilatin, positions 5 and 6 are Gly-Ser, whereas the same positions in rat procardionatrin are Ser-Ala; these two differences in sequence reflect the conserved nature of this peptide in different species.

Cardionatrins II and III are both present in such small amounts in acid extracts of rat atria that it was not possible to characterize them extensively. However, determination of the amino acid sequence of the first 17 residues of cardionatrin III showed that the amino terminus of this molecule is located at residue 73 of the preproprotein. The small amounts of purified cardionatrin II recovered from atrial extracts (~100 pmol per 1000 atria) precluded accurate sequencing of this peptide. Because all

Table 1. Amino acid compositions of cardionatrins I, III, and IV. Compositions were determined by a 24-hour continuous hydrolysis in boiling HCl (6*N*) and analysis on a Beckman 119C amino acid analyzer. Numbers in parentheses indicate the number of residues found in sequence.

Amino acid	Residues (number per mole)		
	I*	III	IV
Asx	2.19 (2)	5.9 (6)	13.3 (14)
Thr		0.97 (1)	3.5 (3)
Ser	4.72 (5)	11.6 (12)	13.4 (15)
Glx	1.39 (1)	4.8 (4)	11.3 (12)
Pro		6.2 (6)	9.6 (10)
Gly	5.03 (5)	10.7 (11)	13.1 (12)
Ala	1.32 (1)	5.2 (5)	8.7 (10)
½ Cys	2.40 (2)	2.3 (2)	1.8 (2)
Val		2.2 (2)	5.9 (6)
Met			1.7 (3)
Ile	2.37 (2)	2.4 (2)	2.2 (2)
Leu	2.16 (2)	9.4 (10)	15.2 (15)
Tyr	1.37 (1)	0.8 (1)	2.6 (2)
Phe	2.28 (2)	2.8 (2)	3.8 (3)
Lys		1.7 (2)	3.1 (4)
His			0.7 (1)
Arg	4.73 (5)	9.5 (10)	8.4 (10)
Total†	28	76	124

*Values taken from (*1*). †Totals for cardionatrins III and IV do not include tryptophan.

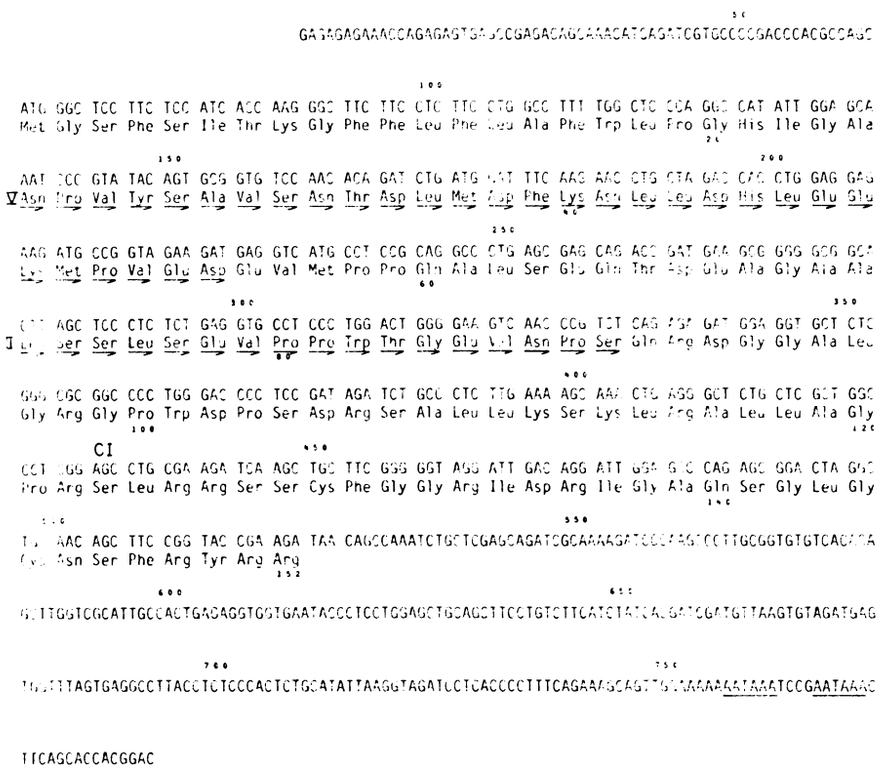

Fig. 3. Preprocardionatrin and its cleavage products. Polyadenylated RNA was isolated from rat atria as described (*20*) and was used to prepare cDNA clones as described (*21*). In this instance, the double-stranded cDNA's were tailed with guanine and annealed into the cytosine-tailed Pst I site of pUC9 (*22*), which was then used to transform *Escherichia coli* strain JM83 to the ampicillin-resistant phenotype. Colonies (650) that remained white in the presence of 5-bromo-4-chloro-3 indolyl-β-D-galactoside were screened for hybridization to the nick-translated ^{32}P-labeled insert from the cardionatrin cDNA clone car 3 (*20*). The DNA from 1 of the 11 clones that hybridized (car 60) was end-labeled with the Klenow fragment of *E. coli* DNA polymerase I and was sequenced (*23*) from the Bgl II sites at 166 and 379 bp (base pairs), from the Ava II site at 367 bp, from the Acc I site at 143 bp, from the Xho I site at 536 bp, from the Cla I site at 657 bp, and from the Hind III and Acc I sites in the multiple-cloning site of pUC9 flanking the cDNA insert. Two putative polyadenylation signals are underlined. The amino terminal sequences of cardionatrins IV and III are indicated by arrows aligned to the preprocardionatrin sequence. These sequences were determined by automatic Edman degradation and by analyzing the phenylthiohydantoin amino acids as described (*24*).

four cardionatrins are natriuretic, it is expected that they contain most of the sequence of cardionatrin I. Compositional analysis of cardionatrins III and IV (Table 1) indicated that these proteins extend up to and include tyrosine at position 150.

Identification of the start points for cardionatrins I and III in procardionatrin confirmed that these products are derived from a common precursor, cardionatrin IV. They are part of a growing list of cleavage products that include a 106-residue peptide (*16*), a 73-residue peptide (*17*), and numerous versions of the carboxyl terminal portion that we have isolated as cardionatrin I. It is not clear which of these are intermediates in a maturation pathway in vivo and which, if any, are artifacts of preparation.

The very small peptides (atriopeptins) isolated and sequenced by Currie *et al.* (*3*) were not present when rat atria were subjected to acid extraction by the procedures we used and by those of de Bold and Flynn (*6*), and it is possible that peptides shorter than cardionatrin (residues 1 to 28) are artifacts of extraction (*18*). Our extraction procedure is similar to that used for the extraction of pituitary hormones by Bennett *et al.* (*8*), who showed that the structural integrity of peptides in rat anterior pituitary was maintained during acid extraction and purification. It is possible, therefore, that cardionatrins I to IV are the natural cleavage products from procardionatrin.

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Leukotriene C_4 Transport by the Choroid Plexus in Vitro

Abstract. *Nanomolar concentrations of peptidoleukotrienes evoke sustained cerebral edema and arterial constriction. Peptidoleukotrienes are thus considered to play an important role in eliciting cerebral edema after cerebral ischemia and vasospasm after subarachnoid hemorrhage. It was hypothesized that the choroid plexus, the locus of the blood–cerebrospinal fluid barrier, might minimize the vasoactivity of locally generated or systemically derived leukotrienes by transporting leukotrienes from cerebrospinal fluid into the blood. Consistent with this hypothesis, leukotriene C_4 in vitro was transported into and released from isolated rabbit choroid plexus by a system that was specific, energy-dependent, probenecid-sensitive, and depressed by cold temperatures. The accumulation of leukotriene C_4 in the choroid plexus was not dependent on tissue binding or metabolism of leukotriene C_4.*

The leukotrienes include the pharmacologically active peptidolipids, one of which, the slow reacting substance of anaphylaxis, is now known to be a mixture of leukotrienes C_4, D_4, and E_4 (*1*). Leukotrienes are synthesized via hydroperoxyeicosatetraenoic acid (HPETE) generated from arachidonic acid by the 5-lipoxygenase pathway (*1*). When leukotriene C is injected intravenously, it does not appear in the central nervous system in functionally relevant quantities (*2*). Whether this is due to poor entry of this large, water-soluble molecule or to active transport of leukotrienes out of the central nervous system, or both, is not clear. However, leukotriene C (LTC_4) is synthesized in gerbil brain after cerebral ischemia and reperfusion (*3*). Moreover, products of the lipoxygenase pathway such as 5-HPETE are detectable in cerebrospinal fluid (CSF) of patients with subarachnoid hemorrhage (*4*). Because of the extremely potent cerebral vasoconstrictor (*5*) and edema-promoting activities (*1*) of the leukotrienes, they have been considered to play an important role in eliciting cerebral edema after cerebral ischemia (*3*) and vasospasm after subarachnoid hemorrhage (*4*).

To eliminate these potent substances, the central nervous system might either metabolize them to inactive products or transfer them to the blood for subsequent metabolism in liver and kidney. We hypothesized that a mechanism for transferring leukotrienes out of the central nervous system might be contained in the CSF compartment within the choroid plexus, a locus of the blood-CSF barrier (*6*). The choroid plexus is involved in the transfer of many water-soluble substances between blood and CSF by separate, specific, carrier-mediated mechanisms (*6, 7*). Our study of the isolated rabbit choroid plexus shows that (i) there is a specific energy-dependent system for uptake of LTC_4 in the choroid plexus, and (ii) this uptake does not depend on binding or intracellular metabolism of the LTC_4, within choroid plexus. Furthermore, the uptake system has a relatively low affinity but high transport capacity for LTC_4. These results are compatible with the choroid plexus serving as a locus of transport of LTC_4 from CSF into blood in vivo.

[14,15-^{3}H]Leukotriene C_4 ([^{3}H]LTC_4; 39 Ci/mmol) (New England Nuclear) was purified by high-performance liquid chromatography (HPLC) before being used (*8*). The choroid plexuses (~5 mg each), obtained from brains of New Zealand White rabbits (1.5 to 2.0 kg) killed with intravenously injected pentobarbital, were individually placed in 3 ml of artificial CSF (in plastic flasks) containing 5 m*M* glucose and 3.0 n*M* [^{3}H]LTC_4 (*9, 10*). In some cases, various other substances were added to the medium. The incubations were carried out in a metabolic shaker at 37°C under 95 percent O_2 and 5 percent CO_2 for various times up to 15 minutes. At the end of the incubation, each choroid plexus was